

Methods for the Analysis of Intestinal Function

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The intestinal tract, an organ of considerable complexity, requires application of numerous techniques for analysis of its physiology and perturbations by toxicologic agents. This review describes the methodology of importance in analysis of the absorptive function of the intestine and the transit of intestinal contents. Methods for studying absorption are categorized according to the technique for administering the test substance such as inclusion in the diet or by gastric and intestinal placement and the method of quantitating the degree of absorption such as determining the appearance of a test substance in systemic fluids or its disappearance from its site of administration in the intestine. *In vitro* techniques which have no *in vivo* analogs, such as the use of the everted sac, are briefly described and their limitations emphasized. Procedures of importance in the clinical diagnosis of malabsorption or in the experimental analysis of absorptive function in man are included and distinguished from techniques used in animal models. In addition, methods for studying aspects of gastrointestinal motility, including the use of luminal markers and analysis of the contractile and electrical activity of intestinal smooth muscle, are reviewed.

The intestinal tract is an organ of considerable complexity, comprising numerous tissue types and subserving multiple functions. The analysis of the physiology of this system has, therefore, involved, and continues to require, the application of research tools from many disciplines within the sciences. For the purposes of this conference, the following review will be restricted to the analysis of the primary function of the gastrointestinal tract, namely the processing of orally ingested substances. Methodology used in studies of various aspects of the digestive function of the gut, the absorptive function, and the contractile properties responsible for transit and elimination of gut contents will be covered, whereas other areas of potential importance to the toxicologist will not. The immunological function of the gut and the techniques used in its analysis will not be reviewed. A discussion of microscopic techniques, including histochemical methods, will also be excluded, although certainly such procedures are critical to analysis of gut function, to validation of experimental manipulations of the gut and to the characterization and elucidation of intestinal diseases and effects of toxic substances.

Studies of Absorption

Study of the absorptive function of the intestinal tract has been carried out with numerous methods. Among the primary considerations in choosing an experimental technique to assess an aspect of the absorption process are the following:

1. The test species to be used: i.e., must the study be conducted in humans with all the accompanying complications or is there an appropriate experimental animal model?
2. The aspect of the absorption process of interest: e.g., is it the overall absorption from the gut lumen to the systemic circulation and tissues, or is it the process of transport across the brush border or basolateral membrane of the intestinal mucosal cell?
3. The experimental or physiological variables to be controlled: e.g., the presence of anesthetic agents, the electrochemical potential difference across the gut wall and the pH of the luminal gut contents.

The answer to these questions will in large part determine the particular method that may be chosen from among the *in vivo* and *in vitro* methods available for studying absorptive function (1, 2). These methods can be categorized according to: the method by which the test substance is administered

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and the method for assessing the extent and/or rate of absorption.

Methods of Administering Test Substance

Various techniques are used for administering the test substance in an *in vivo* study. One is incorporation of the test substance into the diet, which is then administered to the subject. This procedure may be of special relevance to the analysis of the absorption of toxic substances which are contaminants of the diet. A second method is intubation of the test substance into the stomach, a procedure which allows more precise control of the total dose administered. With both these methods the rate and possibly the extent of absorption of the test substance may be markedly affected by the gastric emptying pattern of the subject.

Direct administration of the test substance into the intestinal lumen can also be used; this method eliminates the influence of gastric emptying. In human studies substances can be administered through small-bore intubating tubes localized to particular sites by radiographic techniques (3). In animal experimentation, test substances may be perfused through the gut lumen as a single pass, analogous to the perfusion method in man, or recirculated in the perfusate. Such a technique, illustrated in Figure 1, requires cannulation of the intestine and use of an external heating device and a pump for maintaining the perfusate at body temperature and providing for constant flow.

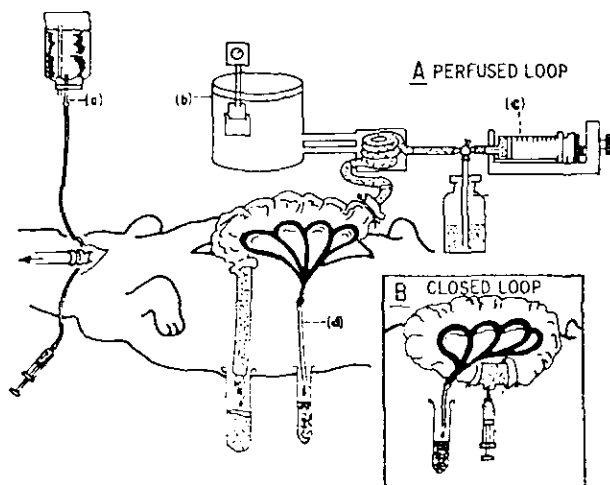


FIGURE 1. Apparatus for perfusion of the small intestine of the rabbit with complete venous collection. Note the constant temperature water bath (b), the perfusion pump (c), and the infusion of blood (a) to permit complete venous collection (d). From Barr and Riegelman (4). Reproduced with permission of the copyright owner.

The following aspects of perfusion techniques deviate from the normal physiologic condition and may affect the absorption process: Animals must be anesthetized although there is a recent publication in which the technique was modified to permit its use in unanesthetized rats (5); The perfusate flushes out the normal luminal contents, such as partially digested materials, biliary or pancreatic secretions; The rate of perfusion through the lumen may differ from the normal pattern of fluid transit and mixing (6).

Another method of direct administration consists of placing a test substance into a segment of the intestine which is closed by ligatures both proximally and distally (2, 7). The construction of the closed segment and injection of the test dose does require the use of anesthetic agents. However, the animal, typically small animals such as rats, can be allowed to recover from anesthesia and to become ambulatory for the majority of the absorption period. This procedure has the additional advantage of not requiring perfusion pumps or heating devices.

Substances may also be administered directly into the intestinal lumen following surgical creation of exteriorized fistulas (1). Studies may then be carried out in unanesthetized larger animals, typically dogs. This approach, of historical importance, has the disadvantage of requiring considerable surgical manipulation.

Methods for Quantitating Degree of Absorption

Appearance in Systemic Fluids. In addition to the method of administering the test substance, the second critical aspect of a technique is the sampling procedure for quantitating the extent and/or rate of absorption. With *in vivo* methods the least invasive techniques entail collection of blood, urine, or breath samples for determination of the appearance of the absorbed test substance and its metabolite in body fluids. Comparison of the time-course of plasma concentrations or excretory rates in urine or breath after oral administration with results after intravenous administration may permit quantitation of the extent of absorption of the test substance and the rate constant of this process (8). This approach is relatively imprecise and may be confounded by numerous factors such as the "first pass" effect (9), the enterohepatic circulation of the agent and the status of elimination processes such as hepatic and renal function. Nevertheless, this technique does have usefulness in the diagnosis of malabsorption syndromes associated with gastrointestinal diseases (10). For example, one test of the transport capacity of the small intestine for carbohydrates entails oral administration of the pentose sugar D-xylose fol-

lowed by its determination in plasma or in urine. Similarly, assessment of intestinal lactase, the disaccharide which cleaves lactose into the absorbable sugars glucose and galactose, involves an oral lactose load followed by determination of blood sugars. A test of fat absorption may include determination of serum carotene. And one test of ileal absorptive function entails the oral administration of radio-labelled Vitamin B₁₂ with determination of its urinary recovery (10).

Sampling of excretory products in breath is an approach for analyzing gut function currently receiving considerable clinical attention (11, 12). This technique entails analysis of breath for hydrogen, generated in the body exclusively by the action of gut bacteria on unabsorbed carbohydrates, or for carbon dioxide, derived from metabolism of an orally administered isotopically labelled test substance. These tests, which can provide an indication of the rate and extent of absorption of a test substance, can be used to detect biliary, pancreatic and mucosal cell malfunction as well as bacterial overgrowth in the small intestine (Table 1). For example, the bile salt glycocholic acid is normally absorbed intact from the ileum and reexcreted in the bile. However, in patients with impaired ileal function or with bacterial overgrowth in the small intestine, there is increased bacterial deconjugation of glycocholic acid with release of glycine. Glycine is then metabolized to CO₂, primarily by bacterial enzymes. Consequently, the administration of glycocholic acid, labeled in the glycine moiety with ¹⁴C, results in an increased excretion of ¹⁴CO₂ in patients with ileal disease or bacterial overgrowth (9). Generally, clinical validation of CO₂ breath tests have been carried out using ¹⁴C radioisotopes. However, use of the stable ¹³C analogs with quantitation of ¹³CO₂ by mass spectroscopy has also been used (13, 14), an approach which may be of special importance for diagnosis of malabsorption in children (15) and women of childbearing age.

The technique of administering an oral load and

Table 1. Breath tests in diagnosis of gastrointestinal function.

Function	Test dose	Analysis
Lactase activity	¹⁴ C-Lactose	Exhaled ¹⁴ CO ₂
Lactase activity	Lactose	Exhaled H ₂
Fat absorption	Triglyceride, e.g., glyceryl esters of ¹³ C or ¹⁴ C triolein	Exhaled ¹³ CO ₂ or ¹⁴ CO ₂
Fat absorption: pancreatic lipase activity	¹⁴ C-Triglyceride and ¹⁴ C free fatty acid	Ratio of exhaled ¹⁴ CO ₂
Bacterial activity	¹⁴ C-Xylose	Exhaled ¹⁴ CO ₂
Bacterial activity	Cholyl-glycine ¹⁴ C	Exhaled ¹⁴ CO ₂

sampling body fluids not only has clinical diagnostic importance but is a useful approach for determination of the overall rate and extent of absorption of environmental contaminants. Such determinations may be important in the theoretical prediction of systemic concentrations of toxic substances following various ingestion rates. Analyses of this sort, referred to by the recently coined term "toxicokinetics", apply mathematical tools extensively used to describe the disposition of pharmacologic agents (8).

A more direct approach for analyzing absorption characteristics than the sampling of systemic or excreted body fluids entails the sampling of portal blood (16) or the collection of the mesenteric blood draining the sites of absorption of the test substance (4), as shown in Figure 1. Such a procedure requires considerably more complicated surgical techniques than sampling of systemic blood, urine or breath. Furthermore, transfusions of blood into the animal may be required. An important advantage of this procedure is the capacity to determine *in vivo* the kinetics of metabolism of a test substance by intestinal tissues. In certain studies, the appearance of the test substance in lymph may be critical, as in the absorption of fats: cannulation of the mesenteric lymphatic vessel may be carried out even in a small animal such as the rat (17).

Disappearance from Intestine. Another approach to quantitating absorption entails monitoring the disappearance of a test substance from the intestine after its administration into the lumen. The gut perfusion method used in humans, for example, monitors differences in the amount infused from the amount appearing at a site distal to the area of infusion. Such perfusion techniques have been exceedingly useful in determination of the transport of electrolytes and nutrients in man. The determination of the amount unabsorbed in the sample taken at a distal site is made possible by the use of a marker substance which is neither metabolized nor absorbed, commonly polyethylene glycol 4000. This particular marker has the additional advantages of lack of adsorption to the gut, high water solubility, stability during frozen storage and ease of determination by radioisotopic or spectrophotometric methods (18). However, one drawback to techniques in which only the luminal contents are sampled is that retention of the test substances in the intestinal mucosa is not quantitated.

In the closed segment procedure, referred to above, degree of absorption is calculated on the basis of disappearance of the test substance from both the lumen and the mucosal tissue. With this method, at the end of the absorption period, the entire segment,

both the gut wall and contents, is assayed quantitatively for the amount of the test substance remaining. One disadvantage of this technique compared to perfusion methods is that sequential samples cannot be taken from a single animal. However, this method is readily used in small animals and can therefore be relatively economical.

In both the perfusion and the closed segment procedures, equating loss of a test substance with its absorption requires verification that disappearance does not result from metabolism in the intestine. If metabolism of the substance does occur, then assay of the intestine alone is inadequate for description of its absorption kinetics, unless the metabolite is poorly absorbed and can be completely recovered in the gut samples.

***In Vitro* Methods**

Numerous advances in our understanding of the cellular mechanisms of electrolyte flux and nutrient absorption in the gut have been made by using *in vitro*, as opposed to *in vivo* techniques. With *in vitro* procedures, physiological variables such as gut motility and mesenteric blood flow can be eliminated or controlled. In addition, the experimenter has the option of control over factors such as the composition of the solutions bathing both the mucosal and the systemic side of the gut and the electrochemical potential difference between the mucosal and serosal side of the gut. *In vitro* techniques include those analogous to *in vivo* methods already discussed. Investigators, for example, have studied the absorption of substances from isolated gut sections with perfusions of the lumen or with perfusions of the vasculature (19).

One *in vitro* method with no *in vivo* analog is the everted sac technique (20). This method has been useful in the characterization of energy-dependent carrier-mediated transport processes. In this procedure small lengths of the intestine are everted, filled with fluid, and tied at both ends. Absorption is quantitated by monitoring the appearance of a test substance inside the sac in the fluid bathing the serosal surface of the gut. Unlike the *in vivo* condition, therefore, absorption of a test substance is considered equivalent, in this model, to its passage not only through the mucosa but also through the submucosa, the external muscle layers and serosal tissue of the gut wall. Modifications have included cannulations permitting sequential sampling of the serosal fluid and control of the serosal fluid volume (21).

Problems with the everted gut segment technique include inadequate oxygen diffusion into the tissue and distension and hydration of the gut segment. Consequently, the preparation of the tissue and the

experimental incubations must be short in duration. Everted sacs of the duodenum from rats exhibit structural abnormalities after 5-min incubation at 37°C (22). By 1 hr, marked distention of the villus as well as complete loss of the villar architecture occurs (Fig. 2). These dramatic structural changes are associated, not surprisingly, with changes in transport kinetics. For example, with this preparation the absorption of large polar molecules such as riboflavin, normally incompletely absorbed from the gut, begins to increase significantly within 30 min (23). One approach to improve tissue viability has been the use of intestinal segments from which the longitudinal and circular muscle layers and serosa have been stripped (24).

Other *in vitro* procedures include rings cut from the whole wall of the intestine (25). This approach is designed to improve oxygenation of the tissue but only permits measurement of the accumulation of test substances by all cell types of the intestine wall. Several other methods have been developed for quantitating uptake of substances specifically by gut mucosal cells. Methods for recovering mucosal cells such as scraping the inner surface of the gut with a glass slide (26) or vibrating a gut segment everted on a glass spiral (27) have recently been improved upon to reduce contamination from cells of the lamina propria (28). In addition, of importance to analysis of carbohydrate digestion and absorption has been the isolation of the mucosal brush border membrane recovered as vesicles following differential centrifugation (29).

An *in vitro* preparation with the advantage of more prolonged viability than cell suspensions is the organ culture of mucosal biopsies (Fig. 3). This technique permits the *in vitro* maintenance of mucosal explants for 24 to 48 hr, depending upon the species and region of the gut biopsied (30). A major advantage of this approach is that the normal anatomical arrangement of the mucosal cells is maintained and the processes of mucosal cell proliferation and differentiation can be studied. Successful applications of this technique have included analyses of hormonal control of mucosal metabolism, proliferation and development and of the biochemical basis of diseases such as celiac sprue.

Studies of Motility

A second major function of the gastrointestinal tract depends upon its contractility. The motility of the gut is responsible for appropriate mixing of orally ingested materials with endogenous secretions required for digestion and for appropriate delivery of substances to the site of absorption or elimination.

One approach to the study of gut motility *in vivo* is



FIGURE 2. Light micrograph of a section of rat small intestine following eversion and incubation at 37°C in Krebs-Henseleit phosphate buffer, pH 7.4, for 60 min. From Levine (2).

the use of an intraluminal marker substance whose transit through the gut lumen can be quantitated. Substances used as valid markers should not be absorbed from the gut, nor adsorbed onto the gut mucosal surface. In addition, ideal markers do not affect any aspect of gut function. Furthermore since the contractile properties of the gut may have different effects on solid as opposed to liquid components of the gut contents, markers should be chosen to correspond to the physical composition of the endogenous substance of greatest interest. This point is of special relevance to the study of gastric emptying (31). Markers used in the tracing of solid substances include, for example, ^{99m}Tc incorporated into a chicken liver meal (31). Markers such as this, being gamma emitters, can be readily monitored for stomach content in humans using a gamma camera.

A technique developed by Summers et al. (32) permits the use of markers to investigate intestinal as opposed to gastric transit in animal models. Permanent, indwelling catheters are surgically implanted in the duodenum of rats and exteriorized behind the head. Markers can, therefore, be administered directly into the small intestine without the disruptive effect of anesthesia or of oral intubation procedures. In animal studies the content of markers in the gut can be determined by analysis of sequential gut segments following sacrifice of the animal.

Other approaches to the *in vivo* analysis of the motor function of the gut (33) include determination of intraluminal pressure changes. These changes can be measured using small balloons or fluid-filled open-tipped tubes connected to external strain gauges, or internal miniaturized strain gauges moni-

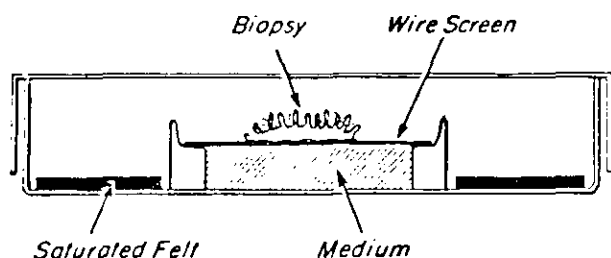


FIGURE 3. Diagram of the technique for culturing intestinal biopsies. The specimen sits on a wire screen over the culture medium. From Trier (30). Reprints by permission from the New England Journal of Medicine.

tored by telemetry or by means of exteriorized wires (34). Analysis of the function of the gastrointestinal musculature may also entail measurement of its electrical activity which in a chronic *in vivo* preparation can be carried out with a recording electrode surgically implanted on or in the gut wall or intubated into the intestinal lumen using a balloon to assure its juxtaposition to the mucosal surface (35).

Conclusion

In analyzing gut function and its perturbations, as in all experimental undertakings, it is critical that the investigator devote considerable attention to the choice of methodology. It is imperative that the limitations of the procedure be carefully considered, as well as factors such as the technical difficulty, the cost, the reproducibility and precision. In addition, successful experimental outcome from application of a chosen method necessitates appropriate use of the tools of experimental design and statistical analysis.

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